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Title: MDGA1, an IgSF molecule containing a MAM domain, heterophilically associates with axon- and muscle-associated binding partners through distinct structural domains.

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Abstract:

Molecules belonging to the immunoglobulin superfamily (IgSF) are reported to be involved in intercellular communication in the developing nervous system. We have identified a novel GPI-anchored IgSF molecule containing a MAM (meprin, A5 protein, PTPμ) domain, named MDGA1, by screening for genes that are expressed by subpopulations of cells in the embryonic chick spinal cord. *MDGA1* is selectively expressed by brachial LMCm motor neurons, some populations of DRG neurons, and interneurons. We found that MDGA1 interacts heterophilically with axon-rich regions, mainly through its MAM domain. Interestingly, MDGA1 also interacts with differentiating muscle through its N-terminal region, which contains Ig domains. These results suggest that MDGA1 functions in MDGA1-expressing nerves en route to and at their target site.

Section: Nervous System Development, Regeneration and Aging

Keywords: spinal neuron, IgSF, MAM domain, muscle

1. Introduction

The formation and establishment of neural circuits is a complex but organized process coordinated by numerous molecules. Fate-committed neurons generated in restricted areas move to their predetermined locations and extend axons to their assigned targets by sensing environmental guidance cues. Immunoglobulin superfamily (IgSF) proteins compose one of the largest families of axon guidance molecules, and are involved in various aspects of the formation and maintenance of neural circuits, largely through modulatory, intercellular interactions (reviewed by Sonderegger 1998, Rougon and Hobert 2003). Combinatorial interactions among IgSF molecules and their cross-talk with other guidance and adhesion systems are major organizing mechanisms underlying the proper formation of neural circuits.

To investigate the development of neural circuits, we screened for genes that are selectively expressed by a subpopulation of chick embryonic spinal neurons, using a single-cell-derived cDNA library (Brady et al., 1990) and suppression PCR-mediated subtraction methods (Diatchenko et al., 1996). Here we report the isolation and initial characterization of one such molecule, MDGA1. MDGA1 is a novel GPI-anchored IgSF protein containing a MAM domain and expressed by a subset of spinal and DRG neurons. MDGA1 interacted heterophilically with axon-rich regions, mainly through its MAM domain, and with differentiating muscle through its Ig-repeat-containing N-terminal region. Our results suggest that MDGA1 may function both en route to and at a neuron's target site, through distinct functional domains, and in cooperation with co-existing molecules.

2. Results

2.1. FY29/MDGA1 is a GPI-anchored extracellular protein selectively expressed by subpopulations of spinal and DRG neurons.

To screen for molecules that could be involved in conferring specific properties to selected neuron types, we screened for genes that are selectively expressed by a specific subpopulation of chick embryonic brachial spinal motor neurons. We have successfully isolated such genes by screening a subtracted library prepared from single-cell-derived cDNAs. This procedure is briefly summarized in Experimental Procedures and the detailed protocol is available upon request. The properties of the other isolated genes will be reported elsewhere.

Of the genes we found, one (FY29) was further characterized because of its unique expression pattern and primary structure. We isolated FY29 from the subtracted cDNA library, which concentrated genes selectively expressed by the LMCm (the medial part of the lateral motor column) motor neurons, which innervate the ventral region of the developing limb. We later found that the human homologue of FY29 was previously isolated as a gene whose expression is up-regulated in some tumors (De Juan et al., 2002), and the HUGO Gene Nomenclature Committee (HGNC) (http://www.gene.ucl.ac.uk/hugo/) has designated the gene *MDGA1*. Hereafter, we call the gene *MDGA1*. Recently, the cloning of its rat homologues and its expression profiles in rat brain were independently reported (Litwack et al., 2004). However, little has been reported on the functional implications of this molecule.

MDGA1 contains six Ig-like domains, one fibronectin-like region, and one MAM domain (Figure 1). Isolation of the mouse ortholog revealed that it is evolutionarily well-conserved (79% identity), suggesting that MDGA1 plays a veiled, yet important, role. As observed in its rat (Litwack et al., 2004) and human (Diaz-Lopez et al., 2005) orthologs, chick MDGA1 was a partially secreted, GPI-anchored extracellular molecule (Supplemental figure 1).

We analyzed the expression and localization of MDGA1 in the embryonic chick brachial spinal cord (Figure 2). Its expression was first observed in the floor plate cells, around Hamburger and Hamilton Stage (HH) 20 (Figure 2A). In post-mitotic neurons, its expression became visible in some motor neurons around HH22 (data not shown), and its selective expression in the LMCm motor neurons were evident by HH24 (Figure 2C). Given that the generation of the brachial LMCm neurons begins by HH20 (Whitelaw and Hollyday, 1983), MDGA1 appeared to be required relatively late in their differentiation, i.e., after their growth cones reached the base of the limb. MDGA1's LMCm-selective expression in brachial motor neurons became more evident by HH27 (Figure 2G) and was retained at later stages (Supplemental figure

2A).

To confirm the LMCm-selective expression of MDGA1, we generated an anti-MDGA1 antibody using a GST-fusion protein of the C-terminal portion of MDGA1 as the antigen (Figure 1). The antibody recognized a single band (Figure 2J) whose molecular mass was comparable to that of rodent MDGA1 (Litwack et al., 2004). Using this antibody, we confirmed MDGA1's LMCm-selective expression in brachial motor neurons by co-staining with an anti-Islet1 antibody that specifically demarcates the LMCm neurons (Tsuchida et al., 1994). As shown in Figure 2L, MDGA1 was selectively observed around the Islet1-positive LMCm nuclei but rarely observed around other nuclei (DAPI-positive), confirming its selective expression in LMCm motor neurons.

MDGA1's expression in regions other than the motor neurons of the spinal cord became visible around HH24 and obvious by HH26, at which time it also appeared to be restricted to certain subpopulations of spinal interneurons (Figure 2G), as observed in rodents (Litwack et al., 2004). That MDGA1 expression was restricted to a specific subset of spinal interneurons was supported by the limited expression of the MDGA1 protein in the ventral funiculus, where only a small subset of the TuJ1-positive axons was labeled with the antibody (Figure 2K, L). This indicated that MDGA1 is selectively expressed by interneurons whose axons run through a relatively restricted path.

In the dorsal root ganglion (DRG), MDGA1 was not expressed by the dorsal DRG neurons (Figure 2G, K), which are TrkA-positive (Lin et al., 1998), suggesting that the majority of the DRG's nociceptive neurons are MDGA1-negative. Of the spinal motor neurons, MDGA1 was not expressed by cervical- and thoracic-level MMC (medial motor column) motor neurons (Supplemental figure 2). The expression of MDGA1 in the lumbar-level motor neurons appeared to be restricted to a subpopulation of LMCm neurons (Supplemental figure 2J), suggesting that MDGA1 may be required for a specific subset of LMCm neurons in the lumbar segment.

2.2. FY29/MDGA1 heterophilically binds central and peripheral axon-rich regions through its MAM domain.

The characterization of MDGA1 as an extracellular GPI-anchored molecule expressed by a specific subset of spinal neurons prompted us to investigate which tissues or cells contained or expressed binding partners for MDGA1. To address this issue, we prepared a soluble AP-fusion protein of MDGA1 lacking the C-terminal region to which GPI is added (919-949; Supplemental figure 1) to use as a probe for potential MDGA1-binding partners. Culture medium containing soluble AP or the AP-MDGA1 fusion protein was overlaid on tissue slices (cryosectioned and later briefly fixed with methanol or TCA to better

preserve the morphology of the tissues following the method described in Takahashi et al. 1997). After extensive washing, the remaining AP activity was examined. As shown in Figure 3, the AP-MDGA1 fusion protein preferentially interacted with the axon-rich regions in the central (Figure 3A) and peripheral (Figure 3D) nervous systems. Interestingly, AP-MDGA1 also interacted with the forming plexus, which is rich with the growth cones of peripheral nerves (Figure 3C). Both fixation methods gave essentially the same results (data not shown). N-terminal and C-terminal AP fusion proteins also gave the same results (data not shown).

MDGA1 contains an Ig domain, and the Ig domains of some other proteins are involved in homophilic interactions. To examine whether the observed binding to axon-rich regions was due to homophilic interactions among the MDGA1s, the same overlay assay was performed using 293T cells expressing MDGA1. No AP-MDGA1 was retained on the MDGA1-expressing cells, although an ephrinA2-AP fusion protein was clearly retained by EphA3-expressing 293T cells (data not shown). To exclude the possibility that MDGA1 might interact homophilically on neurons, the same overlay assay was performed using chick embryos exogenously expressing MDGA1 by *in ovo* electroporation. No preferential interaction of AP-MDGA1 with the MDGA1-expressing regions was observed (Supplemental figure 4B, 4D). In a control experiment, ephrinA2-AP did bind the region where EphA3 was exogenously expressed under the same conditions (Supplemental figure 4A, 4C). Furthermore, AP-MDGA1 clearly appeared to bind to the axon-rich region in the spinal cord, and showed no preferential interaction with the regions where MDGA1 protein was immunologically detected (Figure 3E). These observations strongly support the idea that MDGA1 interacts heterophilically with molecules in axon-rich regions.

MDGA1 contains 3 major structural motifs: 6 tandem immunoglobulin-like repeats, a fibronectin-like region, and a MAM domain. To determine which region is responsible for MDGA1's association with the axon-rich areas, soluble AP-fusion proteins lacking the following putative functional domains were prepared: the MAM domain, the fibronectin-like domain plus the MAM domain, all the Ig domains, and all the Ig domains plus the fibronectin-like domain. Of these mutants, only the AP-fusion proteins lacking the MAM domain (ΔMAM) and all the Ig domains plus the fibronectin-like domain (thus containing only the MAM domain; shown as MAM) were secreted efficiently from the 293T cells; these mutants were used for the overlay assay. The same molar amount of each AP fusion protein was overlaid on tissue slices. As shown in Figure 4, the AP fusion protein of wild-type MDGA1 (SEAP-MDGA1ΔC) bound to the axon-rich regions (SEAP-MDGA1ΔC diluted to match the concentration of the other mutant AP-fusion proteins gave a weaker signal than that observed in Figure 3), and deletion of the MAM domain

greatly reduced the association with the axon-rich regions (Figure 4C). Conversely, an AP-fusion protein containing only the MAM domain had essentially the same association behavior as MDGA1ΔC, although with less binding selectivity (Figure 4D). These results indicate that MDGA1 interacts with the axon-rich regions mainly through its MAM domain.

2.3. FY29/MDGA1 interacts with differentiating muscle through its immunoglobulin-like repeat region.

The expression of MDGA1 in peripheral neurons was sustained until later developmental stages (Supplemental figure 2), when these neurons reached their target muscles, suggesting that MDGA1 might also play a role at these target sites. To investigate whether MDGA1 binding partners were present around muscle tissues late in development, we performed the AP-fusion protein overlay assay using older embryos. Interestingly, AP-MDGA1 preferentially bound to the regions where differentiating muscles reside (Figure 4B and 5C). This interaction was almost certainly heterophilic and *trans*, since *MDGA1* was not expressed by muscle cells or by other cells in the same region (data not shown).

To identify the protein domains of MDGA1 responsible for this interaction, the AP-MDGA1 fusion proteins with the deletions described above were overlaid on embryo sections. In contrast to the interaction with axons, the MDGA1 mutant in which the MAM domain was deleted essentially retained its binding activity to regions of differentiating musculature (Figure 4C and 5D). Conversely, the MAM domain did not interact with these regions (Figure 4D and 5E). The binding partners of MDGA1 resided in virtually all muscles of the forearm (Figure 5C). To determine which remaining motifs of MDGA1, the immunoglobulin-like repeats or the fibronectin-like structure (Figure 1A), were important for its association with muscle, we prepared the mutant lacking the N-terminal 4 Ig-like repeats (Δ4Ig) which was as efficiently produced as the other AP-fusion proteins used in this study. AP-MDGA1Δ4Ig nearly abolished the interaction with developing muscle, even though the axonal binding activity was retained (Supplemental figure 5). This finding indicated that the Ig-repeat region is essential for MDGA1's binding to embryonic muscle. Taken together, our observations suggested that MDGA1 interacts with differentiating muscle heterophilically in *trans* through its N-terminal Ig-like repeats.

3. Discussion

Here we report the characterization of a gene, *FY29/MDGA1*, that is selectively expressed by subpopulations of chick embryonic spinal neurons, including one of the two major subdomains of brachial limb-innervating motor neurons. Its restricted and relatively late onset of expression in the brachial and a subset of the lumbar LMCm neurons suggest that MDGA1 is required for particular events in these limb-innervating motor neurons after they reach the base of the limbs. One prominent event that the LMCm and LMCl neurons undergo at this developmental stage is their bifurcation into ventral (LMCm) and dorsal (LMCl) paths for entering the limbs. *MDGA1* does not appear to be generally involved in this process, because some of the lumbar LMCm neurons apparently do not express *MDGA1*. However, it is still possible that MDGA1 might be required for the proper segregation of these motor neurons at the base of the forelimbs and at a specific choice point in the hindlimbs, since these limbs are differentially specified (Logan, 2003), and each plexus might face somewhat different circumstances. This idea may be supported by the observation that some of our newly isolated genes that are selectively expressed by the brachial LMCm motor neurons exhibit different expression profiles in the lumbar LMC neurons (TY, unpublished observation). It is also plausible that MDGA1 is required for later path-finding and targeting processes, but not at the onset of its expression.

MDGA1 is a GPI-anchored IgSF molecule containing a MAM domain. Its association with axon-rich areas (Figure 3) suggests that MDGA1 might function as an axon guidance molecule in the developing nervous system. More detailed analyses in different experimental systems are necessary to elucidate MDGA1's function. Other observations reported here suggest speculations about the mode of MDGA1's action. MDGA1 interacted with axon-rich regions mainly through its MAM domain, and at least in part, heterophilically (Figure 5; Supplemental figure 4). The MAM domain is found in cell-surface proteins and mediates lateral (*cis*) homophilic interactions in neuropilin-1 (Nakamura et al., 1998) and PTPμ (Cismasiu et al., 2004). Our experiments do not exclude the potential of homophilic association among MDGA1 molecules, that might not be detected by the overlay assay used here. However, the apparent presence of heterophilic binding activity through its MAM domain suggests that MDGA1 might function in an unidentified protein complex. If this interaction is lateral (*cis*), as reported for other MAM domains, the putative interacting molecule could function as a co-receptor and/or modulator. Interestingly, MDGA1 also interacted with differentiating muscle, mainly through its Ig-repeat region in *trans* (Figure 5). This observation suggests that MDGA1 might play a role in targeting the peripheral nerves to muscles as well as in peripheral axonal navigation. It is plausible that the same type of interaction occurs in the central

nervous system. An AP-fusion protein lacking the MAM domain showed greatly diminished binding activity in the axon-rich areas. However, some association, although weak, was observed for this deletion mutant. This observation supports the idea that molecules interacting with MDGA1 in *trans* through its N-terminal region might exist in the axon-rich regions in a relatively diffusive and/or diluted manner. Interestingly, MDGA1 was expressed by floor-plate cells (Figure 2), suggesting that it might also modulate the behavior of commissure axons *in trans*. Combinations of these unidentified MDGA1 binding partners might confer defining properties to specific subtypes of neurons. We are currently identifying these putative interacting molecules, which should help elucidate the molecular details of MDGA1 function.

We independently isolated MDGA1/FY29 as a gene that was differentially regulated in a subtype of motor neurons. MDGA1 was first identified as a gene that was upregulated in some human tumors (De Juan et al., 2002) and was later reported to be differentially expressed in the basilar pons of the rat (Litwack et al., 2004). Including our findings, *MDGA1* has been shown to be differentially expressed among different types of cells, suggesting that it may function to alter some characteristics of the cells that express it, in a relatively general way. Here we report some fundamental characteristics of this molecule that could be informative for analyzing its functions in tumorigenesis and differentiation in the CNS, including in the basilar pons. A recent report showing that MDGA1 is localized to lipid rafts (Diaz-Lopez et al., 2005) may support the idea that MDGA1 modulates the behavior of growth cones, since lipid rafts reportedly mediate chemotropic guidance of growth cones (Guirland et al., 2004). Studies of MDGA1 in different biological phenomena may lead to the unmasking of a previously uncharacterized general mechanism for modulating axon outgrowth and cell motility.

4. Experimental Procedures

4.1. Isolation of chick and mouse MDGA1 cDNA

cDNAs were prepared from single cells obtained from the ventral region of the stage-26 chick (Hamburger and Hamilton, 1951) brachial spinal cord, as in Dulac and Axel (1995). The resultant cDNAs were subjected to suppression PCR-mediated subtraction (in Diatchenko et al., 1996) to create a subtracted cDNA library containing an enriched population of genes selectively expressed by the LMCm (medial part of lateral motor column) motor neurons. The detailed protocol is available upon request. The cDNAs of the subtracted library were isolated, cloned, and analyzed by in situ hybridization performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993; Tsuchida et al., 1994). The cDNA clones that were expressed selectively by LMCm motor neurons were used to isolate longer cDNAs to identify their coding sequences. Of seven newly isolated molecules, one, named FY29, was later discovered to be the chick ortholog of MDGA1. The entire 3' untranslated region was used as a cRNA probe for detecting FY29/MDGA1. Mouse FY29/MDGA1 cDNAs were isolated from a cDNA library made from the brain of a neonatal mouse under low-stringency hybridization and washing conditions, using the coding regions of chick FY29/MDGA1 as the probe.

4.2. Generation of AP-fusion proteins and other expression constructs

Expression plasmids that generated effectively secreted N-terminal AP-fusion proteins were prepared by inserting PCR-amplified fragments of chick FY29/MDGA1 cDNA into the *Xho*I-*Xba*I site of AP-tag5 (Flanagan et al., 2000). Amplified regions were sequenced to confirm that no amino acid changes were introduced. The inserted cDNA fragments were (in bp): 16-918 (wild type; ΔC), 16-739 (ΔMAM), 426-918 (Δ4Ig), and 726-918 (MAM). Respectively, these constructs were full-length except for the last few residues, lacked the MAM (meprin, A5 protein, PTPμ) domain, lacked the first 4 Ig domains, and contained only the MAM domain. A plasmid expressing the N-terminal AP-full-length FY29 was prepared by inserting a cDNA fragment that began with the sequence corresponding to the 16th amino acid residue to the translation stop site into the *Xba*I site of the AP-tag5. The membrane-tethered AP-PDGF transmembrane domain fusion construct was generated by inserting the *Bgl*II-*Xho*I fragment of AP-tag5 into the *BglII-Sal*I site of pDisplay (Invitrogen Carlsbad, CA). An expression plasmid that produced effectively secreted native FY29/MDGA1 (pCAGGSc29ΔC) was generated by inserting the cDNA fragment encoding amino acids 1-918 into the *Xho*I site of pCAGGS (Sunaga et al., 1997). A plasmid expressing the soluble C-terminal AP fusion FY29/MDGA1was generated by inserting the PCR-amplified AP cDNA fragment of AP-tag5 into the *Sal*I site of pCAGGSc29ΔC.

4.3. Generation of anti-FY29/MDGA1 antibody

An expression construct for producing GST-FY29/MDGA1Δ4Ig in *E. coli* was generated by inserting the *Eco*RI-*Xho*I fragment of the AP-fusion protein-expressing plasmid into the *Eco*RI-*Xho*I site of pET42b (Merck, Germany). The purified recombinant proteins were used to immunize guinea pigs. The resultant antisera were affinity purified. Immunohistochemistry was performed as described (Yamada et al., 1993). Donkey secondary antibodies (which show minimal cross-reaction to the serum proteins of other species) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

4.4. AP-fusion protein overlay assay

The AP-fusion proteins were prepared and the AP-fusion protein overlay assay performed essentially as described (Flanagan et al., 2000), with minor modifications. Briefly, AP-fusion proteins were prepared by transfecting 293T cells with AP-fusion protein expression constructs using Lipofectamine 2000 (Invitrogen Carlsbad, CA), following the manufacturer's instructions. The culture medium containing the secreted AP-fusion proteins was collected, and its AP activity was measured using the SEAP Reporter Genes Assay Kit (Roche Diagnostics, Germany). Chick embryos were embedded in Tissue Tek (Sakura Finetech, Torrance CA) and cryosectioned at 20 μm. The sections were fixed in methanol for 10 min at –80ºC and re-hydrated in PBS. Alternatively, the embryos were fixed in 10% TCA for 1 hour at 4ºC, washed with 0.1 M phosphate buffer, equilibrated in 30% sucrose-0.1 M phosphate buffer, embedded in Tissue Tek, and cryosectioned at 12 μm. Both the methanol- and TCA-fixed slices gave essentially the same results. The following procedures were performed at room temperature unless otherwise indicated. The slices were preincubated in 10% FBS-DMEM for 1 hour, and AP-fusion protein-containing culture medium was overlaid on the slices for 2 hours. The AP activity of the medium had been adjusted by dilution with 10% FBS-DMEM. The slices were extensively washed with PBS and fixed with 4% paraformaldehyde-PBS for 20 min. The slices were then washed twice with PBS and incubated in PBS for 1 hour at 65ºC to inactivate the endogenous AP activity. Finally, the slices were washed with 100 mM Tris (pH 9.5) containing 100 mM NaCl and 5 mM MgCl2, and the residual AP activity was visualized by incubating the sections with NBT/BCIP (Roche) in the same buffer for 8-24 hours.

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FIGURE LEGENDS

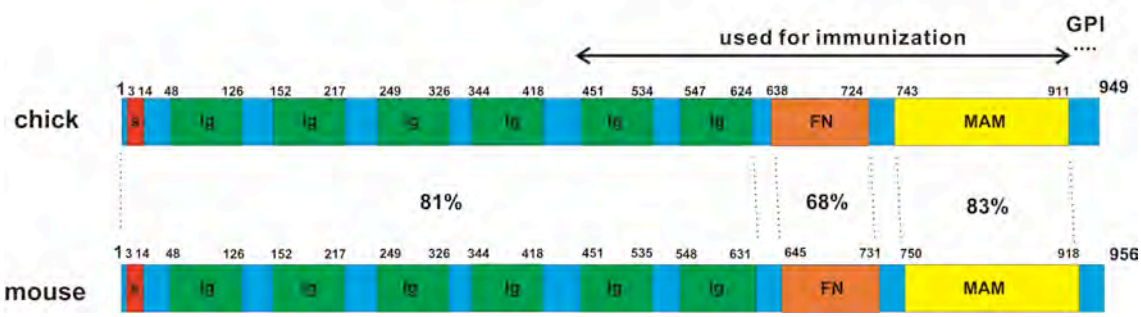


Figure 1. Primary structure of chick and mouse FY29/MDGA1.

Schematic representation of the primary structure of chick and mouse FY29/MDGA1. The percentages of identical amino acid residues between regions of the chick and mouse orthologs are indicated. The portion used to generate the antibody is shown. The C-terminal portion that facilitates the attachment of a GPI anchor is indicated by a dotted line. s, signal sequence; Ig, immunoglobulin-like repeat; FN, fibronectin-like domain; MAM, MAM domain. The nucleotide sequence of chick FY29/MDGA1 has been deposited in the DDBJ/EMBL/GenBank, and its accession number is **AB241390**.

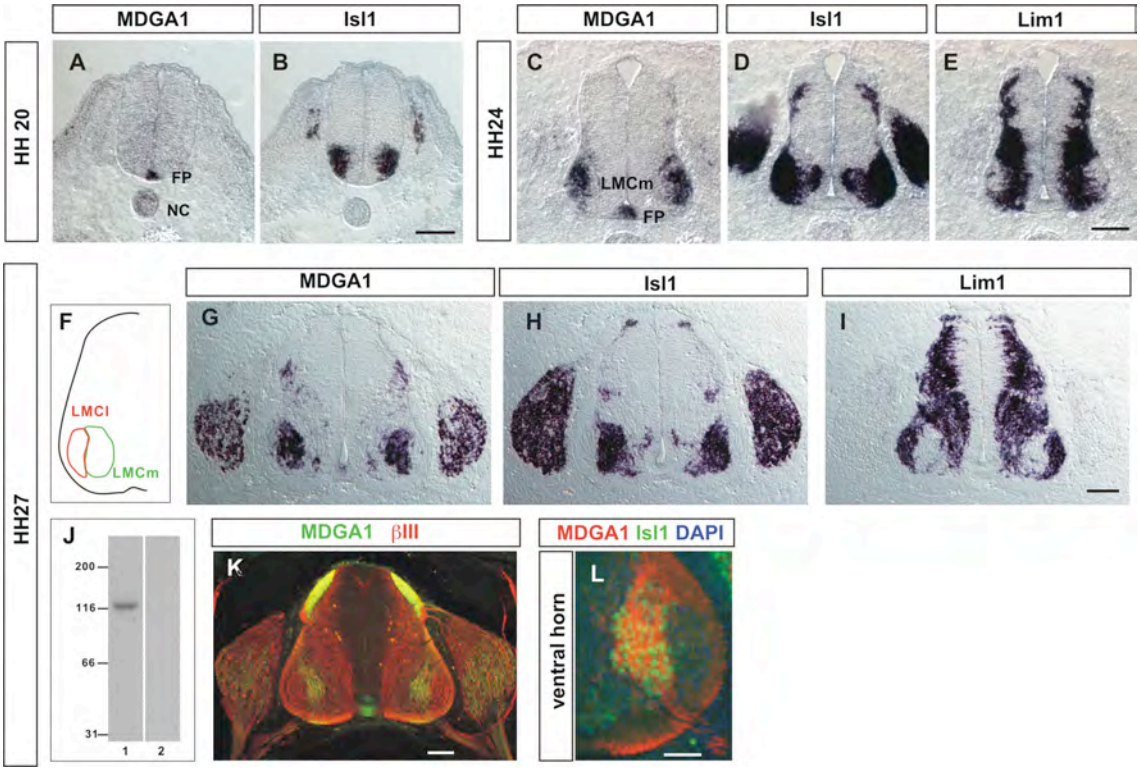


Figure 2. Expression of *MDGA1* in the embryonic chick brachial spinal cord.

A-B, *MDGA1* was not expressed by the early motor neurons. The notochord and floor-plate cells expressed *MDGA1*. C-E, The LMCm-specific expression of *MDGA1* was clearly observed by HH stage 24. F, Location of the LMCm and LMCl motor neurons was demarcated by the expression of *Isl1* (H) and *Lim1* (I). G-I, Expression of *MDGA1* at HH stage 27. MDGA1 was expressed by the LMCm neurons, part of the DRG, and some spinal interneurons.

J, The anti-MDGA1 antibody. An antibody raised against the GST-MDGA1 (426-918) fusion protein recognized a single band in the HH27 spinal cord by Western blotting (J; lane1). This interaction was not observed when the antibody was blocked by the addition of GST-MDGA1 (426-918) (J; lane 2).

K-L, Localization of MDGA1 protein. The indicated proteins were visualized by FITC-anti guinea pig (K), Cy3-anti mouse (K), Cy3-anti guinea pig (L) and FITC-anti mouse (L). MDGA1 was present in the cell bodies and neurites of the Isl1-positive LMCm neurons (K, L) and ventro-medial DRG neurons (K). MDGA1 was selectively observed around the Islet1-positive LMCm nuclei but rarely around other DAPI-positive nuclei (L). In the β–tubulin III-positive axon-rich regions of the ventral funiculus, a small population of axons expressed MDGA1 (K). FP: floor plate. NC: notochord. A-B, C-E, and G-I are consecutive sections. Scale bars: 100 μm (A-I), 50 μm (J).

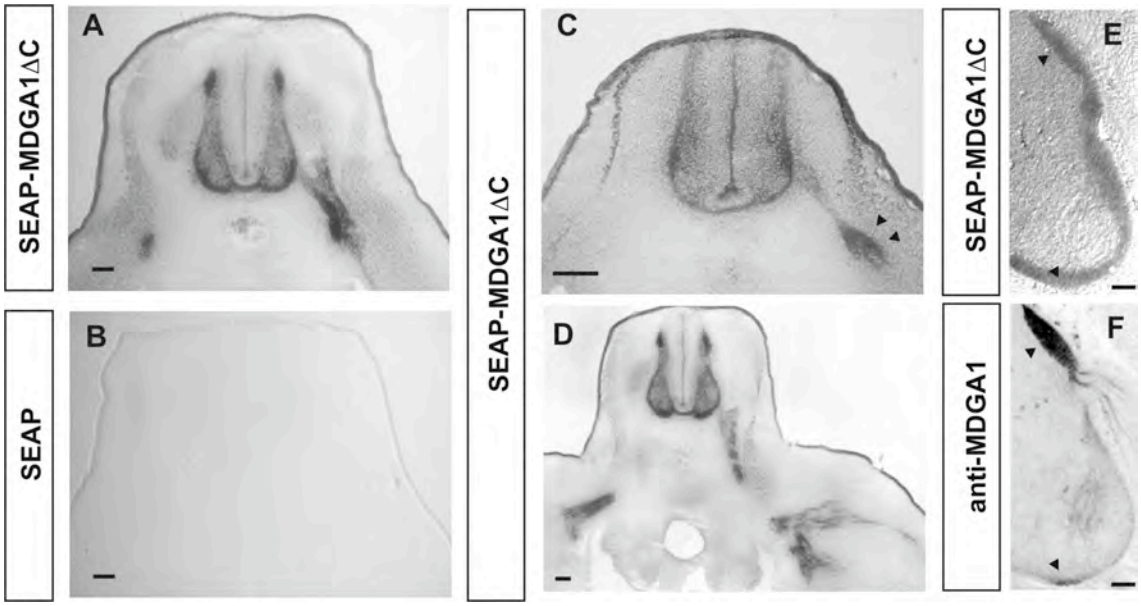


Figure 3. MDGA1 preferentially binds axon-rich regions.

The same molar amount of a secreted AP-fusion protein of MDGA1 (SEAP-MDGA1ΔC) (A, C, D) and AP (SEAP) (B) were overlaid on 20-μm sections from HH24 (A, B), HH22 (C), HH25 (D), and HH27 (E) chick embryos. The AP activities that were retained after extensive washing are shown. No AP activity was detected in the SEAP-overlaid sections (B, data not shown for the other stages). Preferential MDGA1 binding was observed in both peripheral and central axon-rich areas (A, D), including the forming plexus (C, indicated by arrowheads). MDGA1 interacted with the axon-rich regions of both the motor and DRG neurons, including the dorsal root entry zone (A, C). MDGA1 evenly bound the axon-rich regions (E) without any apparent preference for the areas expressing MDGA1 protein (F; indicated by arrow heads). A-B and E-F are adjacent sections. Scale bar: 100 μm (A-D), 50 μm (E-F).

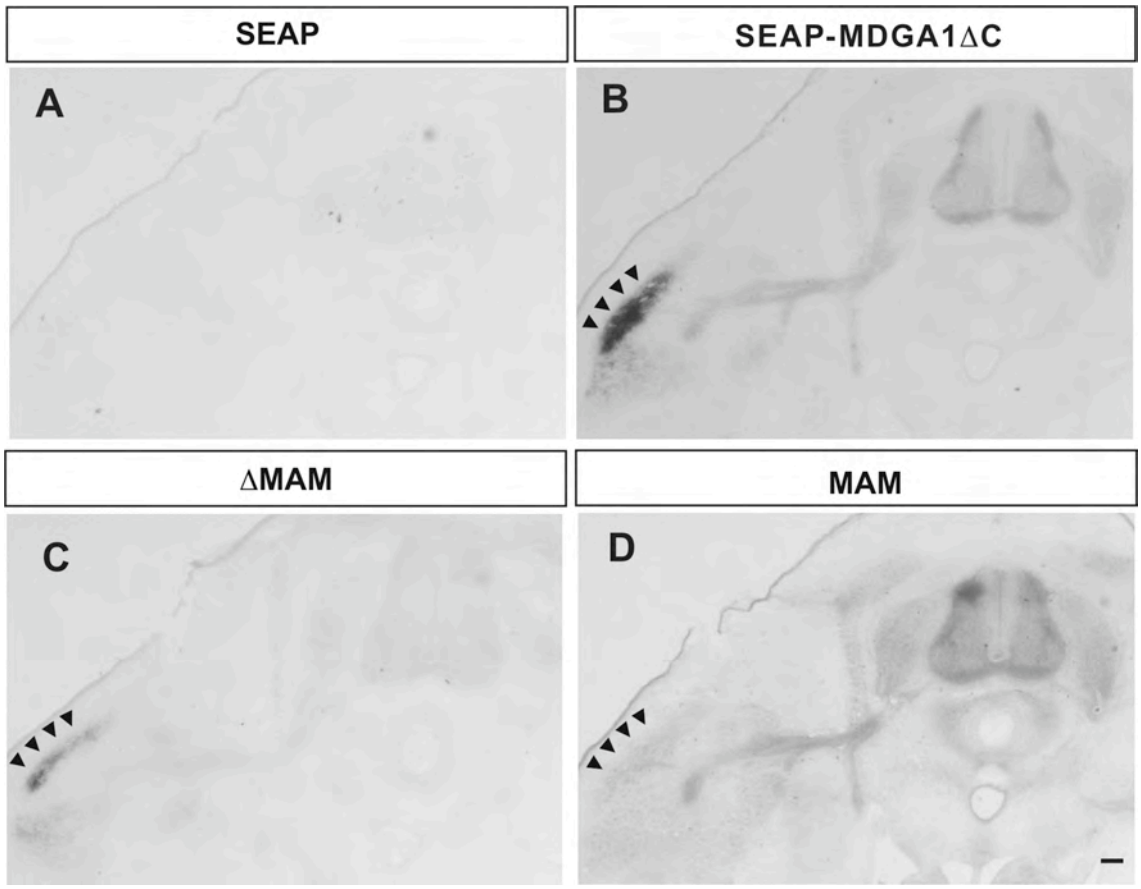


Figure 4. MDGA1 associates with axon-rich regions mainly through its MAM domain.

The same molar amount of AP (A) and the AP-fusion proteins of MAGA1 (B) and its deletion mutants (C, D) were overlaid on 20-μm sections from HH27 chick embryos. Deletion of the MAM domain (ΔMAM) greatly reduced the association with axon-rich regions (C). In contrast, the MAM domain fused to AP (MAM) retained its axon-binding activity with reduced selectivity (D). MDGA1 and ΔMAM associated with differentiating muscle tissue (arrowheads), and MAM retained little of this activity. A-D are consecutive sections. Scale bar: 100 μm.

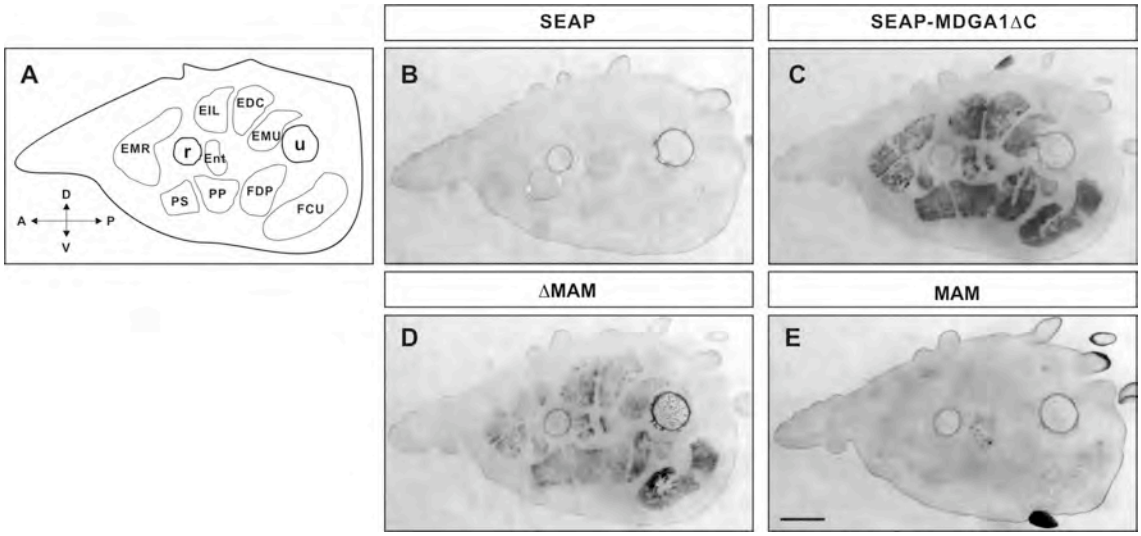
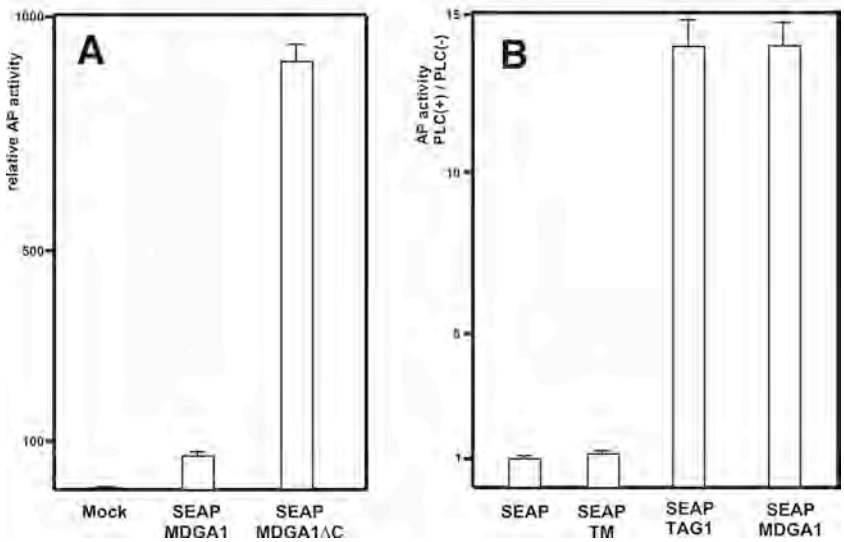


Figure 5. MDGA1 interacts with differentiating muscle mainly through its N-terminal region.

A. Schematic view of the muscle pattern of the forearm at HH36. B-E, The same molar amount of AP (B) and the AP-fusion protein of MDGA1 (C) or its deletion mutants (D, E) were overlaid on 20-μm sections from the forearms of HH36 chick embryos. MDGA1 preferentially interacted with muscle tissues (C). The construct lacking the MAM domain (ΔMAM) retained its muscle-binding activity (D), and the MAM domain fused to AP (MAM) exhibited little association with muscle (E). The muscle identification and nomenclature are according to Robson et al. (1994).

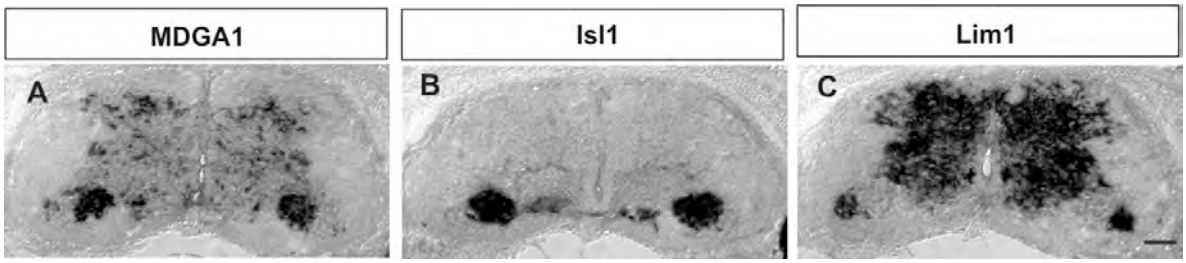
EDC: extensor digitorum communis; EIL: extensor indicis longus; EMR: extensor metacarpi radialis; EMU: extensor metacarpi ulnaris; Ent: entepicondyloulnaris; FCU: flexor carpi ulnaris; FDP: flexor digitorum profundus; PP: pronator profundus; PS: pronator superficialis; r: radius; u: ulna Scale bar: 0.5 mm.



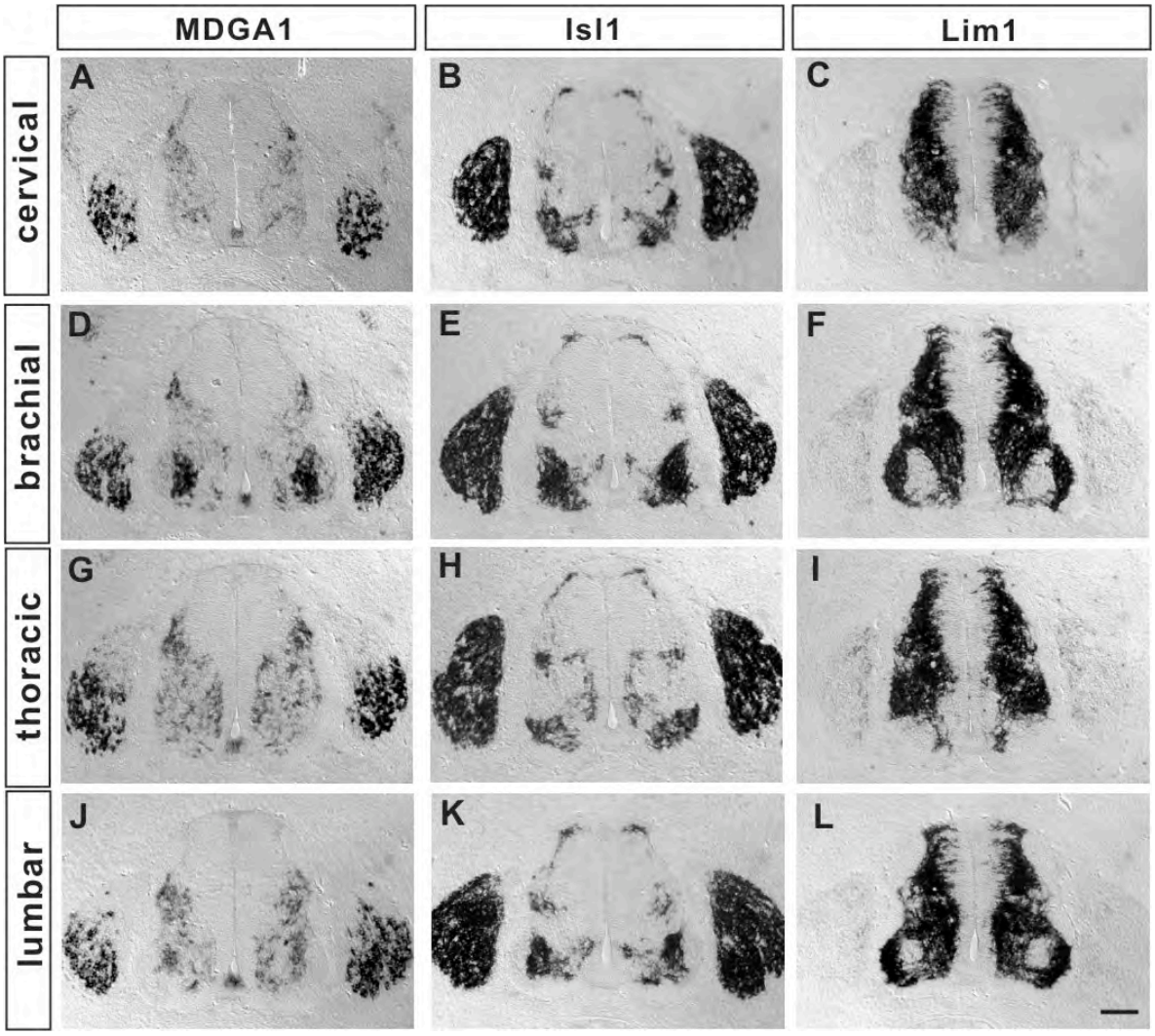
Supplemental figure 1. FY29/MDGA1 is extracellular protein.

A. FY29/MDGA1 is a partly secreted protein. 293T cells were transfected with expression plasmids encoding the N-terminal alkaline phosphatase (AP) fusion protein of MDGA1 (SEAP-MDGA1). The AP activity in the culture medium was measured and normalized to the co-transfected luciferase activity. SEAP-MDGA1 was secreted into the culture medium. This secretion was enhanced by the deletion of its evolutionarily least-conserved C-terminal region (919-949) (SEAP- MDGA1ΔC).

B. FY29/MDGA1 is GPI-anchored. The addition of phosphatidylinositol-specific phospholipase C to the culture liberated SEAP-MDGA1 as well as SEAP-TAG1, an N-terminal AP fusion protein of the GPI-anchored axonal glycoprotein TAG-1. The secreted AP activities from cells expressing the original AP (SEAP) and its PDGF transmembrane domain fusion protein (SEAP-TM) were not altered by the addition of PLC.

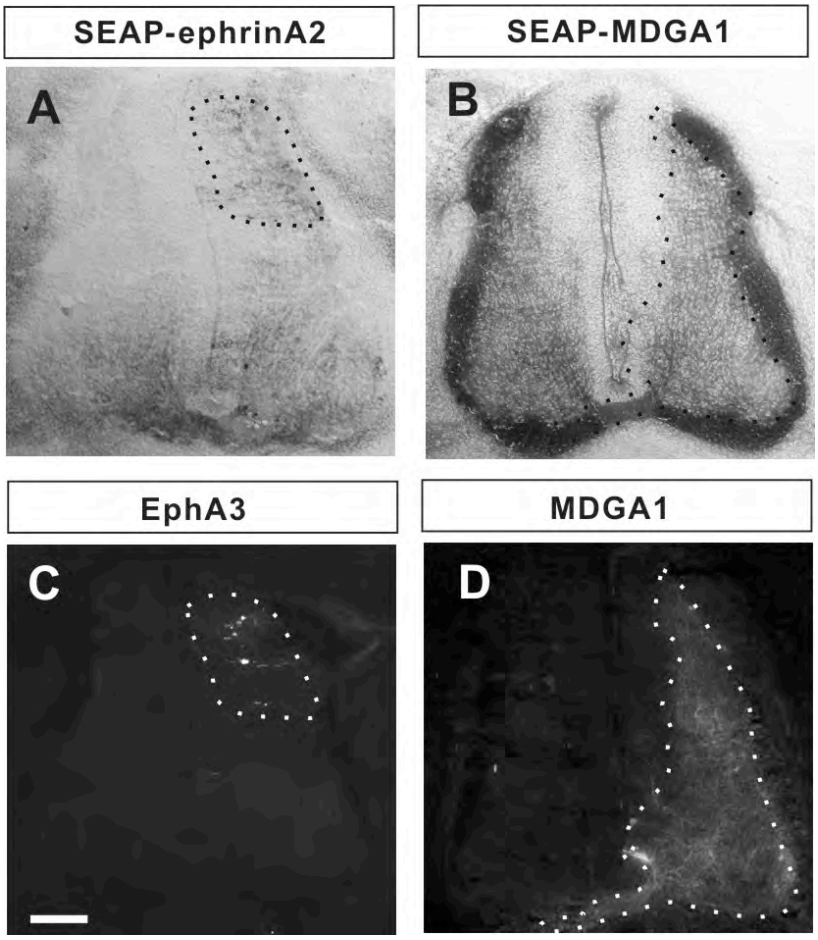


Supplemental figure 2. Expression of *MDGA1* in the chick embryonic brachial spinal cord at HH36. Expression of MDGA1 (A) in Isl1- positive (B) Lim1-negative (C) LMCm motor neurons was retained at HH36. A-C are consecutive sections. Scale bar: 100 μm.



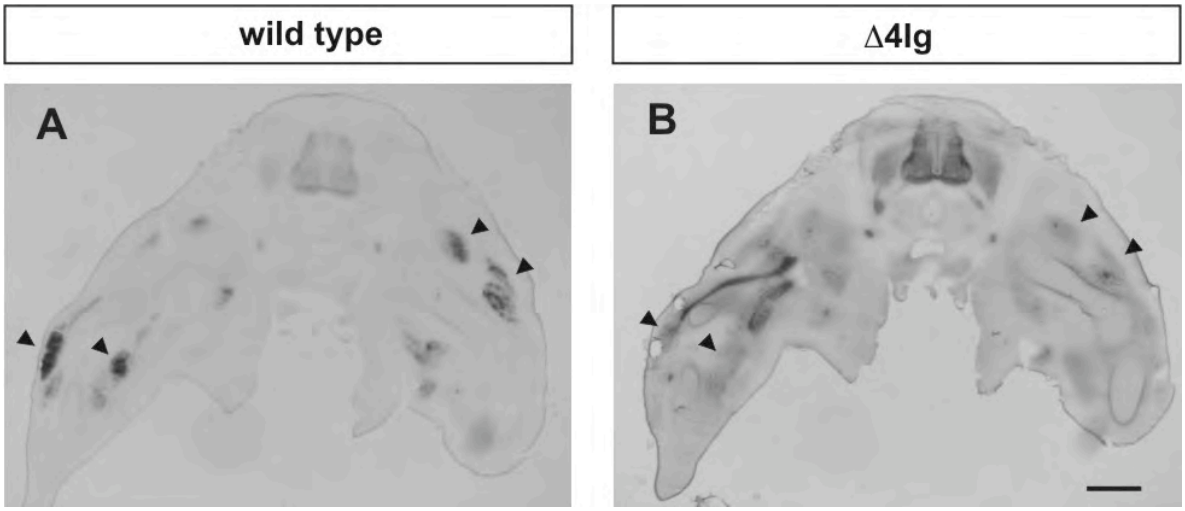
Supplemental figure 3. Expression of *MDGA1* in the chick spinal cord at HH27.

Expression profiles of MDGA1 (A, D, G, and J), Isl1 (B, E, H, and K) and Lim1 (C, F, I, and L) in the cervical (A-C), brachial (D-F), thoracic (G-I) and lumbar (J-L) regions of the chick HH27 spinal cord are shown. Expression of MDGA1 in motor neurons was not observed at the cervical (A) or thoracic (G) levels. In the lumbar region, MDGA1 was expressed by a subpopulation of LMCm motor neurons. The expression pattern of MDGA1 in interneurons was essentially similar throughout the spinal cord with variations in the number of MDGA1-positive cells. A-C, D-E, and J-L are consecutive sections, and I was from a virtually identical rostro-caudal position as G and H. Scale bar: 100 μm.



Supplemental figure 4. Exogenously expressed MDGA1 in the spinal cord does not bind with overlaid MDGA1.

A-B. An AP-fusion protein of ephrinA2 (A) or MDGA1 (B) was overlaid on 20 μm methanol-fixed frozen sections from chick E5 spinal cord that exogenously expressed EphA3 (A) or MDGA1 (B). EphrinA2 bound the area that exogenously expressed EphA3 (C). MDGA1 binding did not increase in the area that MDGA1 was over-expressed (D). Scale bar: 100 μm.



Supplemental figure 5. Deletion of the first 4 Ig-like repeats of FY29/MDGA1 left intact its binding to axon-rich regions, but greatly reduced its association with muscle tissue. AP-MDGA1 (A) or AP-MDGA1 lacking the first 4 Ig-like repeats (Δ4Ig, B) was overlaid on 20-μm methanol-fixed frozen sections from an HH27 embryo. Δ4Ig bound to the periaxonal regions as efficiently as full-length AP-MDGA1, but exhibited greatly reduced binding to muscle tissues (arrowheads). A and B are adjacent sections. Scale bar: 0.5 mm.